Isolation of Crystalline α-Lactalbumin from Milk[2]

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A protein, previously named "crystalline insoluble substance," has been isolated in crystalline form from the albumin fraction of bovine milk whey. The crystalline protein is homogeneous both in the electrophoresis apparatus and in the ultracentrifuge. Its sedimentation and diffusion constants check well with those reported for α-lactalbumin (Kekwick's lactalbumin). There is little doubt that the substances are identical and it is proposed that the protein in milk whey of approximate molecular weight 16,000 and electrophoretic mobility −4.2 at *p*H 8.5 be called α-lactalbumin. This protein comprises about 12% of the total proteins of whey.

Several investigators have reported the isolation of crystalline proteins from the so-called albumin fraction of bovine milk whey.[3] Except for Palmer's β-lactoglobulin and the crystalline albumin isolated by Polis, *et al.*,[4] none of these proteins has been characterized adequately and it has been difficult, if not impossible, to repeat the isolations as originally described. Svedberg and Pedersen in reviewing their studies of whey proteins in the ultracentrifuge[5] attribute the α-peak, one of the three major components in the sedimentation diagram of whey, to a lactalbumin isolated by Kekwick (unpublished). This protein, with constants $s_{20} = 1.9 \times 10^{-13}$, $D_{20} = 10.6 \times 10^{-7}$ and $M_s = 17{,}400$, was referred to as α-lactalbumin.

In 1939, Sørensen and Sørensen prepared from the albumin fraction of whey a crystalline protein which they designated "crystalline insoluble substance"[6] on the basis of its insolubility in water

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(2) A preliminary report of this work was presented at the 1952 Meeting of the American Society of Biological Chemists (*Federation Proc.*, **11**, 220 (1952)).

(3) T. L. McMeekin and B. D. Polis, "Advances in Protein Chemistry," Vol. V, Academic Press, Inc., New York, N. Y., 1949, p. 202.

(4) This protein isolated in trace amounts by B. D. Polis, H. W. Shmukler and J. H. Custer (*J. Biol. Chem.*, **187**, 349 (1950)) was shown to be identical with crystalline bovine serum albumin.

(5) T. Svedberg, *Nature*, **139**, 1051 (1937); T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940, p. 379.

(6) M. Sørensen and S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **23**, 55 (1939).

and in dilute salt solution at *p*H 4.6. The protein was, however, freely soluble in dilute ammonia at *p*H 6–7. "Crystalline insoluble substance" appeared to have the same crystalline form as β-lactoglobulin but it differed from the latter not only in its insolubility in dilute salt solution but in its higher tryptophan content. Neither protein contained phosphorus or carbohydrate. The new protein was not characterized further.

We have prepared "crystalline insoluble substance" along the lines described by Sørensen and Sørensen, and have confirmed many of their observations. In characterizing the protein more completely, it has been found that "crystalline insoluble substance" is homogeneous both in the electrophoresis apparatus and in the ultracentrifuge. Its sedimentation and diffusion constants ($s_{20} = 1.75 \times 10^{-13}$ and $D_{20} = 10.6 \times 10^{-7}$) are in such close agreement with those reported by Svedberg and Pedersen for α-lactalbumin that there can be little doubt that the proteins are identical. It is proposed, therefore, that the protein isolated by the Sørensens also be called α-lactalbumin even though the isoelectric protein is only slightly soluble in water.

Experimental

Preparation of Crystalline α-Lactalbumin.—Casein is separated from 60 l. of raw skimmed milk by the addition of *N* HCl to *p*H 4.6. The whey is adjusted to *p*H 6.0 by means of *N* NH_4OH and then $(NH_4)_2SO_4$ (18.2 kg. for 50.9 l. whey) is added to 2.3 *M* to precipitate the globulin fraction. Sufficient *N* NH_4OH to restore the *p*H to 6.0 is added. The globulin fraction is filtered off. To the clear filtrate (59 l.), 10.0 kg. of $(NH_4)_2SO_4$ is added to 3.3 *M*, the precipitated albumin fraction is filtered off and the filtrate is discarded. The albumin fraction is dissolved in water and dialyzed. When dialysis is complete and the *p*H is then adjusted to 5.2, β-lactoglobulin crystallizes and is centrifuged off. The clear yellow supernatant solution (9 l.) is again brought to 3.3 *M* $(NH_4)_2SO_4$ by the addition of 5.0 kg. of the salt, the precipitated protein is centrifuged off and the mother liquor is discarded. The precipitate is mixed with about 1.5 l. of water and the turbid suspension is again dialyzed at *p*H 5.2. A precipitate is formed as dialysis proceeds. On completion of dialysis, the precipitate, composed of crude α-lactalbumin and some additional β-lactoglobulin, is centrifuged off. The mother liquor still contains much protein which may be recovered and reworked for increased yields. The crude α-lactalbumin precipitate is thrice extracted with small quantities of 0.1 *N* NaCl whereupon the β-lactoglobulin dissolves while the α-lactalbumin does not. The insoluble α-lactalbumin is dissolved in a minimal quantity of 0.03 *N* NH_4OH, the solution is filtered through a thin layer of diatomaceous silica, and the clear filtrate is adjusted to *p*H 4.6 by the addition of 0.1 *N* H_2SO_4. The α-lactalbumin precipitates. It is centrifuged off and again dissolved in 0.03 *N* NH_4OH. The *p*H of the solution is brought to 6.6 (usually a little 0.1 *N* H_2SO_4 is required for this purpose) and to the clear solution an equal volume of saturated $(NH_4)_2SO_4$ solution previously adjusted to *p*H 6.6 with concentrated NH_4OH, is added dropwise with stirring. The precipitate which is formed at half-saturation is amorphous; it is centrifuged off and may be reworked for maximum yields. To the supernatant liquid, the slow addition of saturated $(NH_4)_2SO_4$ solution is continued as before. When the concentration of salt reaches about 58% saturation, crystallization of α-lactalbumin occurs and is complete at about 67% saturation.

Final Purification of α-Lactalbumin.—α-Lactalbumin crystallized at *p*H 6.6 is dissolved in the minimum amount of water, in which it is easily soluble. The protein is reprecipitated by the addition of 0.1 *N* H_2SO_4 to *p*H 4.6 but the product is no longer crystalline. The reprecipitated protein is centrifuged off, dissolved in weak ammonia and again crystallized at *p*H 6.6 as described above. The amounts of amorphous precipitate obtained at half-saturation with $(NH_4)_2SO_4$ diminish with progressive purification. Two or three paired reprecipitations and recrystallizations are required before colorless solutions and uniform crystals are obtained.

To prepare α-lactalbumin in dry form, the recrystallized protein is dissolved in water and reprecipitated at *p*H 4.6 with 0.1 *N* HCl; the suspension is dialyzed until free of chloride ion and then dried from the frozen state. Such drying results in little denaturation because the dried protein may be crystallized again. Four grams of α-lactalbumin is obtained from 60 l. of skimmed milk. The yield is obviously minimal and may be increased considerably by reworking various fractions and mother liquors.

Electrophoretic Properties.—Electrophoretic experiments were run at 0.5° with 1% protein solutions in buffers of ionic strength 0.1. α-Lactalbumin showed only a single boundary at *p*H values 2.0 and 3.0 (glycine–HCl buffers), at *p*H values 6.6 and 7.7 (phosphate buffers), and at *p*H 8.5 (veronal buffer). In contrast to this preponderant evidence for the homogeneity of the protein, an electrophoretic pattern obtained at *p*H 3.3 in lactate buffer showed two components to be present. Further work will be necessary to ascertain whether the inhomogeneity under these conditions is real or an artifact due to some obscure effect of lactate ion. The mobility of α-lactalbumin in veronal buffer at *p*H 8.5 is -4.2×10^{-5} cm.^{2} vol.$^{-1}$ sec.$^{-1}$.

Molecular Weight by Light-Scattering.—M. Halwer of this Laboratory has found that the molecular weight of α-lactalbumin by the light-scattering method[7] is 16,500 (in 0.1 *N* NaCl, *p*H 6.2, 25°).

Ultracentrifuge Experiments.—Measurements of the sedimentation constant of α-lactalbumin in the Spinco ultracentrifuge and of the diffusion constant in the Tiselius cell were made by G. L. Miller and R. H. Golder of The Institute for Cancer Research, Philadelphia. Sedimentation and diffusion runs were carried out at a protein concentration of 1% and in buffer media of 0.18 ionic strength NaCl and 0.02 ionic strength phosphate at *p*H 7.5. The sedimentation runs at 8 and 28°, corrected to 20°, gave $s_{20} = 1.75 \times 10^{-13}$. Measurements of areas under the peaks showed the material to be at least 96% pure. Diffusion measurements at 2°, corrected to 20°, gave $D_{20} = 10.57 \times 10^{-7}$. Theoretical diffusion curves fitted the experimental curves very closely, providing evidence that the diffusion proceeded normally and that the sample was homogeneous.

M. L. Groves of this Laboratory has found the partial specific volume of α-lactalbumin to be 0.735.[8] From this value and Miller and Golder's data, it may be calculated that the molecular weight of the protein is 15,100. The molecular weight of 17,400 given by Svedberg and Pedersen[5] was based on an assumed value of 0.751 for the partial specific volume; if 0.735 is used, M_s becomes 16,400. The discrepancy between 15,100 and 16,400 is of the same order of magnitude as would be expected on the basis of the differences in sedimentation constants recently observed for other proteins by Miller and Golder[9] and by Taylor[10] when measurements made in the Spinco ultracentrifuge were compared with the results of Svedberg and Pedersen.

Solubility Experiments.—In qualitative tests amorphous α-lactalbumin, which had been precipitated at *p*H 4.6 and dialyzed, was found to be largely insoluble in 5% NaCl, 10% NaCl and 70% ethanol. It was easily soluble in 0.1 *N* HCl and in dilute alkali. A few quantitative measurements, preliminary in nature, were made by the technique of Grönwall.[11] The solubility of α-lactalbumin at 25° in either 0.05 or 0.1 *N* NaCl was about 0.2% within the *p*H range of minimum solubility, 4.1–4.8.

Optical Rotation.—α-Lactalbumin was dissolved in dilute NaOH and the concentration of protein was determined by the Kjeldahl method. $[\alpha]^{25}D$ was found to be $-60 \pm 2°$ at *p*H values 6.8, 8.3 and 9.7.

Elementary Analyses.—Table I shows the results of microanalyses performed by C. L. Ogg and his group of this

(7) M. Halwer, G. C. Nutting and B. A. Brice, THIS JOURNAL, **73**, 2786 (1951).

(8) For the method used, see T. L. McMeekin, M. L. Groves and N. J. Hipp, *ibid.*, **71**, 3298 (1949).

(9) G. L. Miller and R. H. Golder, *Arch. Biochem. Biophys.*, **36**, 249 (1952).

(10) J. F. Taylor, *ibid.*, **36**, 357 (1952).

(11) A. Grönwall, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **24**, 185 (1942).

Laboratory. The figures listed have been corrected for moisture and ash, the latter amounting to only 0.1%. Nitrogen was determined by the micro-Kjeldahl method, direct oxygen analyses were made by the Unterzaucher method, and sulfur was determined after Carius digestion. Only trace amounts (0.02%) of phosphorus were found. No carbohydrate could be detected by the orcinol method.[12]

TABLE I

ELEMENTARY COMPOSITION OF α-LACTALBUMIN, %

N	15.86
C	53.32
H	7.01
O	21.56
S	1.91
Total	99.66

Tryptophan Analysis.—Tryptophan was determined by the Spies and Chambers method[13] on the protein in solid form. α-Lactalbumin was found to contain approximately 7% tryptophan. There is some uncertainty about the exact value because the minimum transmittancy of the color produced by this protein was at 620 mμ. Readings at 620 mμ gave a value of 7.2% tryptophan while at 590 mμ, a value of 6.7% was obtained. Sørensen and Sørensen[6] reported that "crystalline insoluble substance" contained 0.371–0.633 mg. tryptophan per mg. protein N (5.9–10.0% tryptophan, calculated on the basis of our determined nitrogen content of 15.86%).

Discussion

The method for preparing α-lactalbumin described above, which was worked out from the observations of Sørensen and Sørensen, has consistently yielded the same crystalline protein. It is of some importance, therefore, to attempt to correlate our findings with some of the earlier studies on the lactalbumin fraction of whey as has already been done with the ultracentrifuge data.

We have been able to prepare α-lactalbumin in crystalline form only in the presence of ammonium sulfate at *p*H 6.6. It has been mentioned that the crystals are easily soluble in water and that when acid is added to *p*H 4.6 to the aqueous solution, amorphous protein, only slightly soluble in water, is precipitated. It may be that the crystalline protein is actually some type of protein–salt combination or simply an ammonium salt and that the crystalline protein isolated by Kekwick[14] was likewise a water-soluble complex and hence called an albumin. The crystalline albumin prepared by Sjögren and Svedberg[15] by the method of Wichmann[16] (salting out with ammonium sulfate in the presence of dilute acid) was also soluble in water. However, the protein was not homogeneous and speculation concerning its solubility is unwarranted.

In regard to the electrophoretic characterization of α-lactalbumin, it is interesting to compare the observed mobility of −4.2 with the data of Smith.[17] In his investigation of the proteins of whey by the electrophoretic method, Smith found that one of the components comprising 12% of the total proteins, had a mobility of −4.5 (protein concentration = 1.23%, veronal buffer *p*H 8.6, ionic strength = 0.1). It seems reasonable to suggest that this component may now be identified as α-lactalbumin.

If the present work has been correlated correctly with Pedersen's ultracentrifuge data and Smith's electrophoretic experiments, it may be concluded that α-lactalbumin is a major component of whey which can be prepared in crystalline form without difficulty. Further studies on its properties and amino acid composition are in progress.

Acknowledgments.—We wish to thank T. L. McMeekin for much helpful advice and for the original suggestion that "crystalline insoluble substance" and α-lactalbumin might be identical. We are also indebted to J. H. Custer for the electrophoretic experiments.

(12) M. Sørensen and G. Haugaard, *Compt. rene. trav. lab. Carlsberg, Ser. chim.*, **19**, 1 (1933).

(13) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

(14) R. A. Kekwick, unpublished; see K. O. Pedersen, *Biochem. J.*, **30**, 948 (1936).

(15) B. Sjögren and T. Svedberg, THIS JOURNAL, **52**, 3650 (1930).

(16) A. Wichmann, *Z. physiol. Chem.*, **27**, 575 (1899).

(17) E. L. Smith, *J. Biol. Chem.*, **165**, 665 (1946).

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